

**MATERIALS  
AND  
METHODS**

## MATERIALS AND METHODS

The biggest challenge for today's microbiologists and medical professionals is to control the spread of potential human pathogens, which have developed resistance to a large number of antibiotics. In order to overcome this problem ethno-biologist together with the microbiologist are looking forward to the higher plants as the source of new, more effective and less toxic antimicrobial substance.

### A. PREPARATION OF PLANT EXTRACTS:

Twenty-eight different medicinal plants and their parts were studied as water, methanol, ethanol and acetone soluble extracts utilizing the techniques mentioned below.

- I. Dried plant materials were ground into powder form. A 3% solution was made in sterile distilled water. It was kept soaked for 3 days and each day it was stirred with a magnetic stirrer for one hour. The mixture was filtered on sterile cheesecloth. The filtrate was divided into two portion, one portion was filter sterilized while the other portion was autoclaved
- II. Fresh plant were washed twice once with tap water then with distilled water. At 4°C Peels from *Beta vulgaris* and *Persea americana* mill were removed with the help of a peeler. Both the peel and the remaining portion of pulp were cut separately into smaller pieces and were soaked in different solvents namely water, ethanol, methanol and chloroform. *Porphyra tenera* consisted

of thin sheets, which were cut into small pieces and soaked in organic solvents and water. From *Solanum tuberosum* three tissues were taken out i.e. peel, sub peel (1/4 cm thick layer from the pulp) and pulp. These tissues were separated and cut into small pieces and soaked in water. The mixtures of organic solvent/water with plant parts were stirred on magnetic stirrer for 1 hr. All mixtures were kept at 4°C for 48 hrs. After two days mixtures were again stirred on magnetic stirrer for 1 hour and were filtered through sterile cheesecloth. Filtrate of each organic solvent was collected in separate evaporating dish. These dishes were kept in fume cupboard for the solvent to evaporate. After two days dried residues were collected. In case of water solution filtrate was centrifuged at 10,000 rpm for 15 minutes, supernatant was collected and was termed as 100% water solution /extract.

## **B. ISOLATION AND IDENTIFICATION OF BACTERIAL CULTURES**

Bacterial cultures used in this study are listed in Table 2. All organisms were grown aerobically at 37°C for 24 hrs on Mueller Hinton agar, except *S. sobrinus* and *S. sanguinis*. *S. sanguinis* was grown anaerobically on tryptic soya broth and *S. sobrinus* was also grown anaerobically but in treated tryptic soya agar / broth the tryptic broth and agar were pre treated with 5 mg of dextranase / gm of the dry mass of the media at 55°C for 2 hrs and 1 mg of invertase/gm dry weight of the media at 37°C for 2 hrs. The bacterial culture was prepared by inoculating 3– 4 isolated colonies from agar plate in the broth tube and incubated in shaking water bath at 37°C for 1.5 hours.

## C. DETERMINATION OF SUSCEPTIBILITY OF BACTERIAL ISOLATES TO STANDARD ANTIBIOTICS AND PLANT PRODUCTS

For the treatment of infectious diseases clinicians select the antibiotic on the basis of their minimal inhibitory concentration (MIC). A traditional method for screening the presence of antimicrobial potential of the plant product/drug is the agar well diffusion method. After confirming the antimicrobial activity of this compound, in this study minimal inhibitory concentration of that product/drug is evaluated by the micro/macro broth dilution method. We have used micro broth dilution method to determine the MIC of the plant-derived substances. Small quantity of the broth culture and the substance is required for the micro method (59).

### I. Agar well diffusion method:

Twenty-four hours old culture was inoculated in Mueller Hinton broth and was incubated for 2-1:2 hr. To determine the actually growing culture Turbidity was then adjusted to 0.5 McFarland. With the help of sterile cotton swab bacterial culture was spread on the Mueller Hinton agar plate. Wells were dug with the help of sterile metallic borer. Different dilutions of the extract prepared in water were poured in respective wells. The plates were incubated at 37° C for 24 hours and zone of inhibition were recorded (18).

### II. Disc diffusion method:

The bacterial cultures (Table 2) were collected from different hospitals and laboratories were first identified according to the standard methods and then

screened against commercially available standard antibiotics (Table 3) for their susceptibility profiles by disk diffusion method of Baur and Kirby.

### **III. Minimum Inhibitory Concentration (MIC) by Micro broth Dilution**

#### **Method:**

In sterile flat-bottomed 96 well plates two fold dilution of aqueous and organic extracts of different plant parts were prepared in Mueller Hinton broth. 20 µl of bacterial culture containing 10<sup>5</sup> CFU/ml was added in each well including positive control containing only broth. Negative control consisted of serial dilution of aqueous and organic extracts only. Plates were incubated at 37°C for 24 hrs and observed for the development of turbidity. The highest dilution of the extract showing no turbidity was recorded as MIC (64.144).

### **D. SCREENING OF PLANT PRODUCTS IN COMBINATION WITH ANTIBIOTICS:**

Large number of antibiotics has lost their efficacy against many bacteria because of the development of drug resistant mutants. To overcome this problem microbiologists are combining two or more antibiotics with different mode of action to prepare the synergistic combination. In this study two synergistic combinations have been formulated, one containing amoxicillin and water solution of *C. fistula* and other consists of Shamimin and amoxicillin. The synergistic preparations were evaluated by the following methods.

## I. Disc Diffusion Method

Filter paper discs were divided into two groups. Disc in-group 1 were impregnated with 10 µl of the combination (leaf stem or fruit), group 2 had aqueous fruit extract of *C. fistula* (150 µg/ 10 µl) or amoxicillin (15 µg/ 10 µl) alone. Leaf, stem and fruit extracts of *C. fistula* were tested for the synergistic activity by the disc diffusion assay (131). Bacterial inoculum of MDR *S. typhi* was prepared by suspending them in Mueller Hinton Broth from MacConkeys agar plate. Broth culture was incubated for 2-½ hrs, turbidity was adjusted to 0.5 McFarland standard. 15 minutes of adjusting the turbidity of the inoculum, with the help of the sterile cotton swabs the dried surface of the Mueller Hinton agar plate was streaked with the organisms. Filter paper discs were evenly placed and firmly pressed on the inoculated plate with the help of forceps. After 24 hours of incubation, the antimicrobial effects of the samples that produced zone of inhibition were recorded (18).

## II. Checkerboard Titration

Combination of fruit extract with Amoxicillin gave synergistic effect against the multidrug resistant strains of *S. typhi*. Checkerboard titration was carried out to determine the synergy and MIC of Amoxicillin and fruit extract of *C. fistula* in micro titer trays with cation supplemented Mueller Hinton broth (Difco USA). Fruit extract of *C. fistula* and Amoxicillin were tested at five different concentration from 195 – 3120 µg/ml and 0.39 – 12.5 µg/ml respectively. Fruit extract of *C. fistula* was dispensed alone in the first row, *C. fistula* and Amoxicillin were combined in the remaining row, and amoxicillin was also dispensed alone in the last column. Inocula were prepared by

suspending growth from MacConkeys agar plate in to Mueller Hinton broth. Density of broth culture at logarithmic phase was adjusted to that of 0.5 McFarland Standard. This was diluted such that final inoculum contained  $5 \times 10^5$  CFU/ml. Trays were incubated aerobically over night. The lowest dilution containing no turbidity was considered as MIC. Fractional Inhibitory Concentration (FIC) were calculated as MIC of the amoxicillin and fruit extract of *C. fistula* in combination / MIC of the amoxicillin or fruit extract of *C. fistula* alone (Table --). In order to evaluate the outcome of combination of Amoxicillin and fruit extract of *C. fistula* fractional inhibitory (FIC) index was calculated as the summation of  $FIC = FIC_{\text{amoxicillin}} + FIC_{\text{C. fistula}}$ . Individual checkerboard was repeated three times for each isolate and combination. A mean FIC index was calculated practical to a commonly utilized definition of synergy and classified as either synergistic ( $\leq 0.5$ ) Additive or indifferent ( $\geq 0.6-2$ ) antagonistic ( $0.2 \rightarrow 4$ ) (39,231).

#### **E. EFFECT OF PLANT PRODUCTS ON GROWTH KINETICS OF SUSCEPTIBLE BACTERIA**

In order to determine the bactericidal effect of plant derived substances on the growth of bacteria. 1/10 of MIC of Amoxy-cassia, *C.fistula* and Amoxieillin were incorporated in the broth tubes separately. Each tube was inoculated with bacterial culture containing  $10^5$  CFU/ml. Number of Cell forming unit (CFU) were counted at 0<sup>th</sup> minute, 30 minutes and then after each hour till 8 hours. Graph was plotted between log of number of bacteria versus time (241).

## F. EFFECT OF PLANT PRODUCTS ON THE PATHOGENESIS OF CARIOGENIC BACTERIA

For the initiation of infectious diseases, the most important factor is the adhesion of bacteria to the host tissues. Oral bacteria cause infections due to their ability to adhere on the teeth and other surface of the oral cavity, aggregation of the bacteria to the product dextran produced by *S mutant* species and coaggregation among different bacterial pathogens of the oral cavity.

In this study the bacterial species used were *S.sobrinus*, *S.sanguinis*, and *A.naeshundii* T14V. The organisms were pretreated with organic extracts at the concentrations of 25 mg/ml and 2.5 mg/ml and 50% and 5% of aqueous solution of *Persea americana mill*, *Beta vulgaris* (beet) root, *Porphyra tenera* (sea vegetable) and *Solanum tuberosum* (potato). Three models were set up to study the effect of plant products on infection of oral bacteria, which are as follows:

1. The first model consists of adherence of cariogenic *S. sanguinis* to human 'O' RBC. In this assay eleven two-fold dilutions of pretreated *S sanguinis* at  $1.0 \times 10^8$  were prepared in sterile 96 well plates. Well number 12 was treated as negative control. In all wells 20 $\mu$ l of 20% human 'O' RBC were added. In positive control plate mannose sugar was also included along with bacteria and RBC. The plates were incubated at 37°C for 2 hours. Plates were observed for the formation of matrix/button. To confirm the results plates were incubated at 4°C for 24 hours and observation was recorded again.

- II. In the second model aggregation of *S.sobrinus* with high molecular weight dextran (produced by *Strept. mutans*) was studied using 96 well microtiter plates. First well was kept as control containing 0.065 ml of untreated culture. The remaining wells contained 0.065 ml of pretreated cultures of *S. sobrinus*. Then 0.020 ml of distilled water and finally 0.005 ml of high molecular weight dextran (0.5 µg/ml) was added in each well. OD of the wells was recorded at '0' minute and then after every 30 minutes for 2 hours. Graph was plotted between the OD of the culture and time in minutes. In negative control experiment low molecular weight dextran was included to inhibit the aggregation. Decrease in OD was observed in case of positive control (56).
- III. In the third model *A. naeshlundii* T14V co- aggregates with *S. sanguis* 35 when present in oral cavity. *In- vitro* similar assay was set up in 96 well microtiter plates. *A. naeshlundii* T14V was treated with the plant derived substances. The first well of microtiter plate was kept as positive control and 50µl of *S sanguis* and untreated *A. naeshlundii* T14V at OD 1.0  $A_{540}$  were added. In remaining wells *S. sanguis* 35 and pretreated *A. naeshlundii* T14V were added and OD was observed at '0' minute and after every 30 minutes for 2 hrs. In negative control plate β-lactose [β-x-Gal (1-4)] was added to inhibit the reaction.

**G. EFFECT OF PLANT PRODUCTS ON THE ATTACHMENT OF UROPATHOGENIC *E. COLI* TYPE I.**

Mannose resistant *E. coli* type 1 attaches itself to the cells of the urinary tract lining with the help of pili. The pili have the ability to produce the capsule like substance, which covers the bacteria, and make them indistinguishable by the immune system. During this study, we developed an in-vitro model to determine the adherence of mannose resistant *E. coli* type 1. Mannose resistant *E. coli* type 1 was allowed to adhere to the guinea pig RBCs. In this assay eleven two-fold dilutions of pretreated mannose resistant *E. coli* type 1 at an OD 1.0  $A_{540}$  were prepared in sterile 96 well plates. Well number 12 was treated as control, which contained saline. In all other wells 20 $\mu$ l of 20% guinea pig RBC was added. In positive control plate mannose sugar was also included along with bacteria and RBC. The plates were incubated at 37°C for 2 hours. Plates were observed for the formation of matrix/button. To confirm the results plates were incubated at 4°C for 24 hours and results were recorded again.

## II. FLOW CYTOMETRIC EVALUATION OF THE EFFECT OF PLANT PRODUCTS ON CARIOGENIC AND UROPATHOGENIC BACTERIA

Most important step in the pathogenesis of bacteria is its ability to adhere to the host surface. Because after firmly attaching themselves to the host tissue, bacteria secrete toxic substances and multiply in the body of the host. Thus plant derived substances that inhibits the adherence of bacteria to the host play an important role in treatment of infectious disease. Earlier in this study, in-vitro adhesion of cariogenic *S. sanguinis* and mannose resistant *E. coli* type 1 were studied in micro titer 96 well plate. The plant extracts, which were found to be effective in inhibiting bacterial adherence to human RBC thus reducing hemagglutination titer as compared to the control, were

further evaluated by the flow cytometry technique. Bacterial cultures of *S. sanguis* and *E. coli type 1* were grown for 18 hours in tryptic soya broth then centrifuged at 4°C and 10,000 rpm for 10 minutes. The bacterial culture was washed twice with PBS 7.3. The optimal density of the culture was adjusted to density 1.0 at  $A_{540}$ . The bacterial cultures were pretreated with aqueous solution and organic extracts of the plant. Bacterial culture used as control was left untreated. Fluorescent staining of bacteria was performed according to Logen et al. Fluorescent labeled *S. sanguis* were mixed with human 'O' RBC and *E. coli type 1* with guinea pig RBC.

The reaction was analyzed on Fluorescence Activated Cell Sorter ( FACS - Becton – Dickinson, Sparks, MD) Flow cytometric analyses were performed using cell quest software provided by the manufacturers (Becton Dickinson- USA ). Scattered plots consist of points representing each RBC, where the 'X' axis is a measure of the size of detected particles and the Y-axis measures internal cellular complexity i.e. (granularity). Histogram indicates the distribution of RBC based on their relative fluorescence intensity and Y is the number of particles counted at specific fluorescence and set as a basal of fluorescence resulting in histogram peak at the far left of the X axis is the number of particles counted at the specific fluorescence intensity. For histogram analysis MI marker was set to incorporate approximately 95% of the events for the auto fluorescence of RBC (Fig. 1-A). The median fluorescence intensity of subsequent histogram peaks falling outside. The median fluorescence intensity of subsequent histogram peaks falling outside the MI marker was recorded for adhesion analysis as an increase in overall Fluorescence.

When analyzing the adhesion between bacteria and RBC bacterial clumps occasionally appeared with the size range of the RBC. The clump did not interfere with the analysis of the RBC due to their ability to exclude this data from analysis of RBC fluorescence intensity. The clump bacteria can be disregarded by running a sample of bacteria alone and gating out the resulting dot plot population therefore selecting a population that contained a majority of RBC. Furthermore histogram data representing clumped bacteria can be excluded from analysis by setting an additional marker around the clumped bacteria peak and disregarding any data that appears within this peak (which always appear outside the range of RBC intensity).

#### **I. EFFECT OF PLANT PRODUCTS ON *S. AGALACTIAE* VAGINAL INFECTIONS IN RATS.**

*S. agalactiae* are known to colonise in the female vagina, though they don't cause any harm to the normal adult. But when they are present in pregnant women they are transferred to the baby during delivery and can be a cause of fatal neonatal diseases. The plant products used to develop this infection model were polyphenoloxidase (PPO) and asparaginase. Both of these plant-derived enzymes are known to have the property of inhibiting the adhesion of bacteria to the eukaryotic cells.

For this infection model, virgin female Sprague Dawley rats (source) were infected with the inoculum containing  $10^7$  *S. agalactiae*. Infection was allowed to develop for 2 days. They were then divided into three groups, first group was treated with 80 units of asparaginase second group was treated with 200 units of PPO and the control group

was treated with saline. Again after 12 hours same treatment was given to each group of rats. Each day from day '0' to day '20', 10 µl of vaginal secretion was collected and was cultured on Columbia blood agar with 5% sheep blood supplemented with 10 µg/ml colistin sulfate and 15 µg/ml of nalidixic acid for the growth of *S. agalactiae*. Number of colony forming units (CFU) of *S. agalactiae* 10µl of vaginal secretion in each rat were counted and recorded (122).

#### **J. TOXICITY OF PLANT PRODUCTS FOR BALB/c MICE**

In order to evaluate the toxicity level of various drugs. Lethal dose (LD<sub>50</sub>) is usually determined in mice and other laboratory animals by exposing them to several different concentrations. In this study the plant extracts were administered in different doses to check their toxicity in BALB/c mice. Different group of mice A, B, C, D were injected by the intra peritoneal route with different dilutions of plant extracts. The group E that received only saline instead of plant products was included as control group. Animals in group A were injected intra-peritoneal with 1000, 500, 250 and 125 mg of aqueous fruit extract / Kg body weight of mice. Group B was given 100, 50, 25 and 12.5 mg of amoxicillin / body weight of the mice. Group C was given combination of fruit extract and amoxicillin in the concentration of 1000:100, 500:50, 250:25 and 125:12.5 mg/body weight of the mice. Group D was given 150,300,600, 1200mg of methanol extract of *P.harmala*. Animals were kept under observation and their mortality and behavior was observed up to two weeks. The LD<sub>50</sub> was calculated by Reed and Muench method. (23.115).

#### **K. EFFECT OF PLANT PRODUCTS ON HOST IMMUNE RESPONSE**

There are many plant derived substance and antimicrobial compounds, which affects the immune system. Some of them have positive effect on immune system as they enhance the activity of the specific and non-specific immune response to infection. They can play a very effective role in the treatment of different infectious diseases specially in immunocompromized patients. Immunomodulating properties of our synergistic formulation "Amoxy-cassia", and each component i.e. *Cassia fistula* and amoxicillin was studied by evaluating the antibody titer and the number of antibody secreting plasm cells in BALB/c mice. Assays employed were Haemagglutination and Hemolytic plaque assay.

In this experiment, 4 groups' 20 female BALB/c mice were used. Each group consisted of 5 mice each. On day zero, first group of mice were intra-peritoneally injected with 10 µg/ml of Amoxy- cassia. Group 2 were treated with 10 µg/ml of *Cassia fistula*. Group 3 mice were treated with 1 µg/ml of amoxicillin. Group 4 animals were treated with saline. On day '1' same treatment as day '0' was given to each animal and then 0.2 ml of 10% washed SRBCs were injected in the peritoneum of group 1, 2, 3 and 4. On day 2, in group '1' animals, 5 µg of Amoxy- cassia was injected intra peritoneally. In group 2 mice, 5 µg/ml of *C fistula* fruit solution was introduced. In group 3 animals 0.5 µg/ml of amoxicillin was introduced. The control group of animals, Group 4 was treated with saline instead of Amoxy-cassia. Effect of plant-derived substance on the immune system of BALB/c mice was determined by anti- SRBC hemagglutination titer in test and control animals.

#### **I. Anti- SRBC antibody titer by haemagglutination (H.A)**

Blood from the treated and control mice were collected on day 4, 5, 6 and 7. Serum was separated. Twenty-three, two fold serial dilutions of serum from each group of mice were prepared in microtiter plate. The contents of the wells were mixed thoroughly. Then 20  $\mu$ l of 1% sheep RBC were added in each well. Plates were incubated at 37°C for 2 hrs. And then wells were observed for matrix /button formation. Plates were incubated again at 4°C over night to confirm the results. Reciprocal of the highest dilution showing hemagglutination was considered as hemagglutination titer (IIA).

## **II. Hemolytic plaque assay for detecting anti-SRBC antibody secreting plasma cells**

In this experiment each the animal was killed on day '4' by cervical dislocation and the spleen was removed aseptically. The spleen was macerated and washed in tissue culture medium. Then the cells were filtered and centrifuged. The pellets were diluted in the tissue culture and cell density was adjusted to  $10^5$  cells/ml. Spleen cells were stored on ice.

The spleen cells were mixed with equal volume of 100 $\mu$ l ml of washed 10% SRBC and 300 $\mu$ l of plating medium (1% agarose in Hanks balanced salt). The suspension was kept in water bath at 50°C and was mix thoroughly. Spleen cell mixture was poured on to the pre- coated glass slides (0.1% agarose coated). Slides were incubated at 37°C for one hour. Then 8 ml of 1:2 diluted guinea pig or rabbit complement in veronal saline was poured on the slide and incubated for 30 minutes at 37°C. Number of plaques/ $10^5$  cells were counted.

To confirm the results slides were incubated at 4 °C over night and plaque were counted again (211).